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Reduced response to adiponectin and lower abundance of adiponectin receptor proteins in type 2 diabetic monocytes

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Abstract **The abundance of the adiponectin receptors, AdipoR1 and AdipoR2, and the effects of the antidiabetic adipokine adiponectin in monocytes of normal-weight and overweight controls and type 2 diabetic patients (T2D) were analyzed. AdipoR1 and AdipoR2 mRNAs were increased in monocytes of obese controls and T2D patients when compared to normal-weight controls, and AdipoR1 mRNA positively correlated to AdipoR2 mRNA, the waist to hip ratio and systemic adiponectin. However, AdipoR1 and AdipoR2 proteins were lower in monocytes of T2D compared to normal-weight donors. Induction of IL-6 and IL-8 by adiponectin, an effect involving p38 MAPK, was also reduced in T2D monocytes.**
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Keywords: Adiponectin; Monocyte; Type 2 diabetes; Obesity

1. Introduction

Adiponectin is an adipocyte-derived adipokine with antidiabetic, and antiatherosclerotic properties of which systemic levels are reduced in obesity, type 2 diabetes and coronary artery disease [1–3]. In vitro studies show that adiponectin induces the cell death of myelomonocytic progenitor cells, reduces foam cell formation in macrophages and prevents the adhesion of monocytes to TNF-activated endothelial cells [4,5]. In addition, adiponectin influences the secretion of cytokines and chemokines. The LPS-stimulated release of the multifunctional cytokine IL-6 is reduced in macrophages [6,7], whereas in non-activated cells an induction of IL-6 has been reported [6,8]. Furthermore, adiponectin inhibits IL-8 secretion of human macrophages co-cultivated with apoptotic cells, whereas the IL-8 production of LPS-activated macrophages was rather augmented by adiponectin irrespective of the presence or absence of apoptotic cells [9]. An elevated release of IL-8 by adiponectin is also found in non-activated macrophages [8,10] and cytokine-mediated IL-8 release is not inhibited by adiponectin treatment [10]. Therefore, adiponectin should rather be considered to function as an immunoregulator than an antiinflammatory protein.

Adiponectin activity depends on the adiponectin receptors 1 and 2 and the mRNAs of these receptors are expressed in monocytes [11,12]. In ob/ob mice, AdipoR1 and AdipoR2 mRNAs are reduced in skeletal muscle and adipose tissue [13]. In human skeletal muscle of first degree relatives of type 2 diabetes patients [14], the mRNA expression of these receptors is also lower. High-fat feeding, however, increases hepatic AdipoR1 and AdipoR2 mRNA and muscle AdipoR1 in mice [15].

The insulin/phosphoinositide 3-kinase/Foxo1 pathway regulates AdipoR1 and AdipoR2 mRNA expression and whereas fasting and insulin deficiency elevate the mRNA levels, insulin replenishment and feeding reduces AdipoR1 and AdipoR2 in the liver and skeletal muscle of mice [13]. AdipoR1 is involved in adiponectin-mediated phosphorylation of the AMP kinase and induction of IL-6 secretion by the p38 MAPK pathway, whereas AdipoR2 is important for the activation of peroxisome proliferator activated receptor-α [11,16].

Most of the data published so far indicate that circulating adiponectin and the mRNA expression of AdipoR1 and AdipoR2 are reduced in insulin resistance postulating a state of adiponectin resistance in glucose intolerant animals and humans. Therefore, in the current study AdipoR1 and AdipoR2 mRNA and protein and the response to adiponectin were analyzed in monocytes of normal-weight controls, overweight probands and type 2 diabetes patients.

2. Materials and methods

2.1. Material

RPMI medium was obtained from Gibco BRL (Karlsruhe, Germany). Recombinant human adiponectin expressed in a mammalian cell line, DuoSet ELISA Development Systems for human adiponectin, IL-10, IL-8 and IL-6 were from R&D Systems (Wiesbaden-Nordenstadt, Germany), Vacutainer CPT were from Becton Dickinson (Franklin Lakes, NJ). ApoE antibody was from Chemicon (Hampshire, UK) and CD163 antibody from Serotec (Düsseldorf, Germany). The antibodies were used at a 1:1000 dilution for immunoblots. The p38 MAPK inhibitor SB 203580 was from Calbiochem-Merck (Darmstadt, Germany). The cytotoxicity detection kit was from Roche (Penzberg, Germany). AdipoR1 antibody was generated as recently described [17] and AdipoR2 antibody was ordered from EBL (Hamburg, Germany) and used at a dilution of 1:100.

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2.2. Subjects

The study protocol was approved by the local ethics committee and the investigation conformed with the principles outlined in the Declaration of Helsinki (1997). Each proband gave written informed consent to participate in the study.

Monocytes of 11 male T2D patients, 11 male controls with a waist to hip ratio (WHR) above 1 (CG1) and 11 male controls with a WHR below 1 (CG2) were isolated. Details of the study groups are given in Table 1.

T2D patients were treated with metformin, glinides, glitazones, statins, fibrates, acarbose, aspirin, sulfonylureas and 4 patients were insulin dependent. The probands of the CG1 group were treated with RAAS inhibitors, aspirin, proton pump inhibitors, doxazosin, or enalapril and 5 probands had no medication. Probands of the CG2 group were not on medication.

2.3. Isolation and culture of primary blood monocytes

Peripheral blood leukocytes were isolated from 32 ml of whole blood by Vacutainer CPT and monocytes were further purified by magnetic separation with CD14 beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Serum was coagulated with Thromborel S (Roche Diagnostics GmbH, Mannheim, Germany) and $CaCl_2$ and was 3-times dialyzed against PBS for 2 h. Monocytes (3×10^6) were cultivated in 1 ml RPMI medium supplemented with 10% autologous serum for 24 h. Subsequently, the medium was replaced. Monocytes were either cultivated in RPMI supplemented with 10% autologous serum or in the identical medium supplemented with 10 μg/ml adiponectin. Supernatants were collected 24 h later and used for ELISA. MAPK inhibitor was added 1 h before the addition of adiponectin.

2.4. Real-time RT-PCR

Real-time RT-PCR was performed as recently described [17]. The specificity of the PCRs was confirmed by sequencing of the PCR fragments. Primers for AdipoR1 and AdipoR2 were used as previously published [17]. The primers for β-actin were 5′-CTA CGT CGC CCT GGA CTT CGA GC-3′ (β-actin uni), and 5′-GAT GGA GCC GCC GAT CCA CAC GG-3′ (β-actin rev). IL-6 was amplified with IL-6 uni (5′-GCC CAG CTA TGA ACT CCT TCT-3′) and IL-6 rev (5′-GCG GCT ACA TCT TTG GAA TC-3′) and IL-8 was amplified with IL-8 uni (5′-ACC GGA AGG AAC CAT CTC ACT G-3′) and IL-8 rev (5′-GCA TCT GGC AAC CCT ACA ACA-3′).

2.5. ELISA

The ELISAs were performed as recommended by the distributor. Supernatants were used undiluted for IL-10 determination. For IL-8 ELISA supernatants of control-treated monocytes were diluted 1:1000 and of adiponectin incubated cells 1:5000 and 1:10 or 1:100, respectively, for IL-6 ELISA. EDTA-plasma was diluted 1:5000 to determine adiponectin. All measurements were performed in duplicate.

2.6. SDS–PAGE and immunoblotting

Monocytes (3×10^6) were washed with PBS and solubilized in 50 μl RIPA buffer. Fifteen micrograms of protein was separated by SDS–polyacrylamide gel electrophoresis and transferred to PVDF membranes (Bio-Rad, München, Germany). Incubations with antibodies were performed in 1% BSA in PBS, 0.1% Tween overnight. Detection of the immune complexes was carried out with the ECL Western blot detection system (Amersham Pharmacia, Deisenhofen, Germany).

2.7. Statistics

Data are given as the median values and the range of the values (SPSS 12.0 for Windows). Data are represented as Box Plots indicating the median, the upper and lower quartile, the largest and the lowest value in the data set. Statistical differences were analyzed by two-tailed Mann–Whitney *U*-Test or Wilcoxon Test for paired samples and a value of $P < 0.05$ was taken as statistically significant. The Pearson's correlation was calculated using the SPSS 12.0 software.

3. Results

3.1. AdipoR1 and AdipoR2 mRNA and protein in monocytes of controls and T2D patients

Monocytes were isolated from normal-weight controls (CG2), overweight donors (CG1) and T2D patients and the AdipoR1 and AdipoR2 mRNA levels were analyzed. The expression of AdipoR1 mRNA normalized to β-actin was 0.63 (0.28–1.23) in CG2, 1.11 (0.75–4.49) in CG1 and 2.47 (0.42–4.70) in T2D monocytes. Monocytes of the CG1 and of T2D patients expressed similar levels of AdipoR1 mRNA and significantly higher levels compared to CG2 ($P = 0.001$ for CG1 and T2D, respectively) (Fig. 1A). AdipoR2 mRNA in CG2 was 0.66 (0.24–1.14) thus being significantly higher in CG1 with 0.96 (0.59–2.14) ($P = 0.008$) and T2D monocytes with 0.88 (0.34–2.07) ($P = 0.036$). Expression of AdipoR2 mRNA was similar in monocytes of T2D patients and CG1 (Fig. 1B). AdipoR1 mRNA positively correlated to the BMI ($r = 0.43$, $P = 0.012$), the waist circumference ($r = 0.5$, $P = 0.003$), and the waist to hip ratio (WHR) ($r = 0.53$, $P = 0.002$) (Fig. 1C), whereas AdipoR2 mRNA did not correlate to these anthropometrical factors (not shown). In addition, there was a positive correlation of the AdipoR1 to the AdipoR2 mRNA level ($r = 0.61$, $P = 0.0001$) when expression in the monocytes of all probands was considered (Fig. 1D). AdipoR1 and AdipoR2 protein were analyzed by immunoblot in monocytes of 6 T2D patients and 6 normal-weight controls and the results of 3 probands of each group are shown in Fig. 1E. Quantification of the immunoblots and normalization to β-actin levels revealed that AdipoR1 protein was 9.7 (7.4–12.0) in CG2 and 7.0 (5.8–7.1) in T2D cells ($P = 0.004$). AdipoR2 was 6.3 (3.2–10.4) in CG2 and 2.9 (0.3–4.0) in T2D monocytes ($P = 0.01$).

Table 1
Anthropometrical and biochemical characteristics of the study groups (only males)

	T2D	CG1	CG2	*P*-value
N	11	11	11	ns
Age (years)	60.5 (42.0–76.0)	57.0 (40.0–72.0)	56.0 (44.0–67.0)	ns
BMI (kg/m^2)	33.0 (27.2–45.9)	30.1 (27.7–36.5)	23.6 (20.0–27.7)	0.0001[a], 0.0001[b]
WHR	1.06 (0.91–1.11)	1.04 (0.99–1.13)	0.93 (0.83–0.98)	0.0001[a], 0.0001[b]
WC (cm)	111 (108–133)	110 (95–118)	105 (86–113)	0.01[b]
HDL (mg/dl)	45.0 (35.0–71.0)	42.8 (24.2–61.0)	51.7 (39.1–78.0)	0.016[b]
TG (mg/dl)	149.5 (88.0–737.0)	113.8 (78.8–381.5)	89.5 (49.0–224.0)	0.014[a]
Cholesterol (mg/dl)	175.1 (126.0–265.0)	167.4 (115.2–228.0)	189.0 (145.8–230.4)	ns
Adiponectin (μg/ml)	1.65 (0.58–3.84)	2.29 (1.00–5.60)	2.22 (1.16–4.18)	ns

Control group 1 (CG1), control group 2 (CG2), body mass index (BMI), waist circumference (WC), waist to hip ratio (WHR), triglycerides (TG).
[a]Significance of T2D versus CG2.
[b]Significance of CG1 versus CG2.

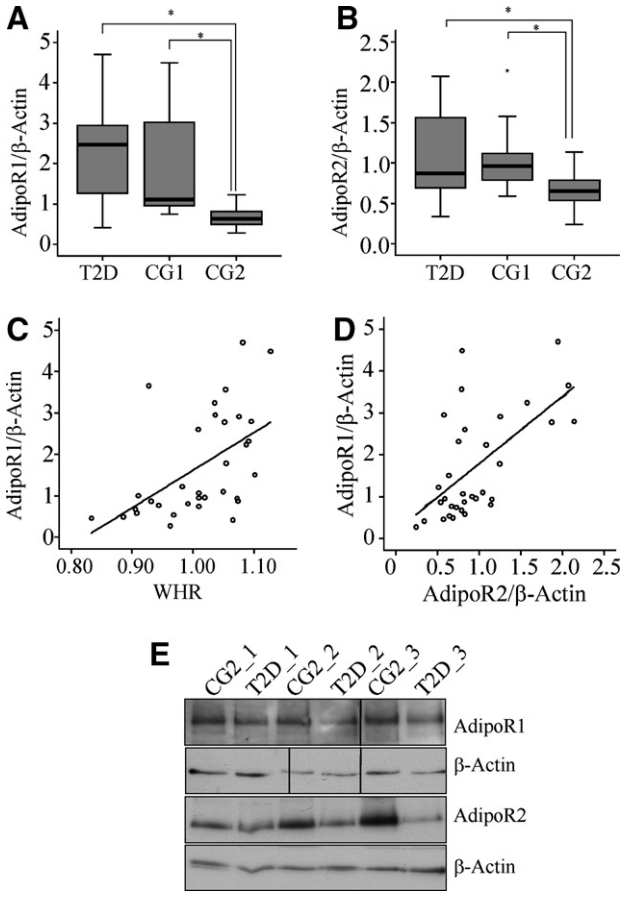

Fig. 1. AdipoR1 and AdipoR2 in monocytes of type 2 diabetes patients (T2D), overweight controls (CG1) and normal-weight controls (CG2) and correlation to the waist to hip ratio (WHR). (A) AdipoR1 mRNA was determined by real-time RT-PCR in monocytes of the three groups mentioned above and the expression was normalized to β-actin mRNA levels. (B) AdipoR2 mRNA was determined by real-time RT-PCR in monocytes of the three groups mentioned above and was normalized to β-actin mRNA levels. (C) Correlation of monocytic AdipoR1 mRNA to the WHR. (D) Correlation of monocytic AdipoR1 and AdipoR2 mRNAs. (E) AdipoR1 and AdipoR2 protein in monocytes of three different CG2 (CG1 to CG3) and three different T2D (T2D_1 to T2D_3) donors. $^{*}P < 0.05$.

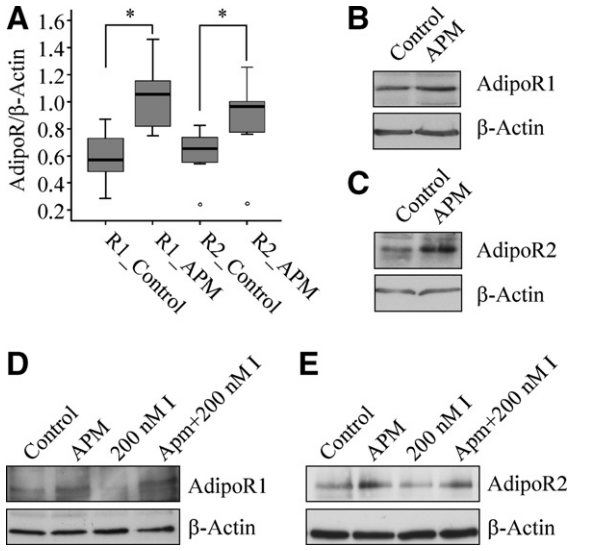

Fig. 2. Adiponectin-mediated induction of AdipoR1 and AdipoR2 in primary monocytes. (A) Monocytes of eight healthy individuals were incubated with 10 μg/ml adiponectin for 24 h and AdipoR1 and AdipoR2 mRNA expression normalized to β-actin mRNA is shown. The circles indicate outliers. (B) Monocytes were cultivated as described in (A) and AdipoR1 and β-actin were analyzed by immunoblot. (C) Monocytes were cultivated as described in (A) and AdipoR2 and β-actin were analyzed by immunoblot. Monocytes of healthy individuals were incubated with 10 μg/ml adiponectin for 24 h alone, with 200 nM p38 MAPK inhibitor (I) or adiponectin in combination with 200 nM p38 MAPK inhibitor and AdipoR1 (D) and AdipoR2 (E) were determined. $^{*}P < 0.05$.

3.2. Induction of AdipoR1 and AdipoR2 by adiponectin in vitro

AdipoR1 and AdipoR2 mRNA were measured in monocytes of 8 normal-weight individuals cultivated without or with 10 μg/ml adiponectin for 24 h. AdipoR1 mRNA normalized to β-actin mRNA was 0.57 (0.28–0.87) in control-incubated cells and was induced to 1.05 (0.75–1.45) by adiponectin ($P = 0.001$). AdipoR2 mRNA was 0.65 (0.24–0.83) in the control cells and was also significantly ($P = 0.007$) upregulated to 0.96 (0.25–1.26) by adiponectin (Fig. 2A). However, there was only a weak positive correlation of AdipoR1 ($r = 0.41$, $P = 0.018$) and no correlation of AdipoR2 mRNA ($r = 0.34$, $P = 0.05$) with systemic adiponectin levels (not shown). Immunoblot was performed with monocytes isolated of three different normal-weight donors that were incubated with 10 μg/ml adiponectin for 24 h and the result of a representative experiment is shown in Fig. 2B and C. AdipoR1 protein and AdipoR2 protein were detected in the monocytes by immunoblot and were slightly upregulated (1.5-fold (1.3–1.7) for AdipoR1 and 2.1-fold (1.8–2.7) for AdipoR2) by adiponectin in vitro. However, an induction of the receptors was not observed when monocytes of 5 T2D patients were analyzed (not shown).

Adiponectin activates the p38 MAPK and therefore the effect of adiponectin on AdipoR1 and AdipoR2 induction was determined in monocytes pretreated with the p38 MAPK inhibitor SB 203580 at a concentration of 200 nM. Upregulation of the receptors was not influenced by this inhibitor (Fig. 2D and E).

3.3. Adiponectin upregulates IL-6 and IL-8 via the p38 MAPK pathway

We and others have recently demonstrated that adiponectin enhances IL-6 and IL-8 release in monocytes [6,9,10]. However, it has not been analyzed whether the mRNA expression of these proteins is induced. Abundance of IL-6 mRNA in monocytes of the 8 normal-weight individuals was 0.40 (0.07–2.55) and was upregulated by adiponectin to 22.6 (3.3–49.2) ($P = 0.03$). IL-8 mRNA in the monocytes was 0.37 (0.05–1.23) and was increased by adiponectin to 9.51 (1.91–45.4) ($P = 0.02$) indicating that the transcription or the mRNA stability is enhanced by adiponectin (not shown).

Because p38 MAPK may be involved in the induction of these proteins, monocytes of three healthy controls (three independent experiments per donor) were incubated with 10 μg/ml adiponectin alone or in combination with the p38 MAPK inhibitor SB 203580 at a concentration of 100 nM or 200 nM. IL-6 secretion from control-incubated cells was 256.0 (162.1–265.0) pg/ml and 100 nM or 200 nM inhibitor

did not significantly influence IL-6 release. Adiponectin upregulated IL-6 to 1984 (1897–2075) pg/ml ($P = 0.001$ versus control). 100 nM of the inhibitor reduced adiponectin-stimulated IL-6 release to 1122 (523–1420) pg/ml ($P = 0.007$ versus adiponectin stimulated cells) and 200 nM of the inhibitor lowered secretion of IL-6 to 821 (272–892) pg/ml ($P < 0.001$ versus adiponectin-stimulation) (Fig. 3A). Basal IL-8 release was 30.1 (22.7–34.8) ng/ml and in adiponectin treated monocytes 88.7 (84.4–98.1) ng/ml ($P < 0.001$ when compared to monocytes not incubated with adiponectin). 100 nM SB 203580 did not alter IL-8 whereas 200 nM of the inhibitor lowered basal IL-8 to 21.6 (18.7–24.6) ng/ml ($P = 0.002$ versus control). Adiponectin induced IL-8 was reduced by 100 nM of SB 203580 to 69.1 (56.2–78.8) ng/ml and by 200 nM SB 203580 to 60.4 (48.9–79.5) ng/ml ($P < 0.001$ versus adiponectin-treated cells for both concentrations) (Fig. 3B) indicating that p38 MAPK signaling is important for IL-6 and IL-8 induction by adiponectin.

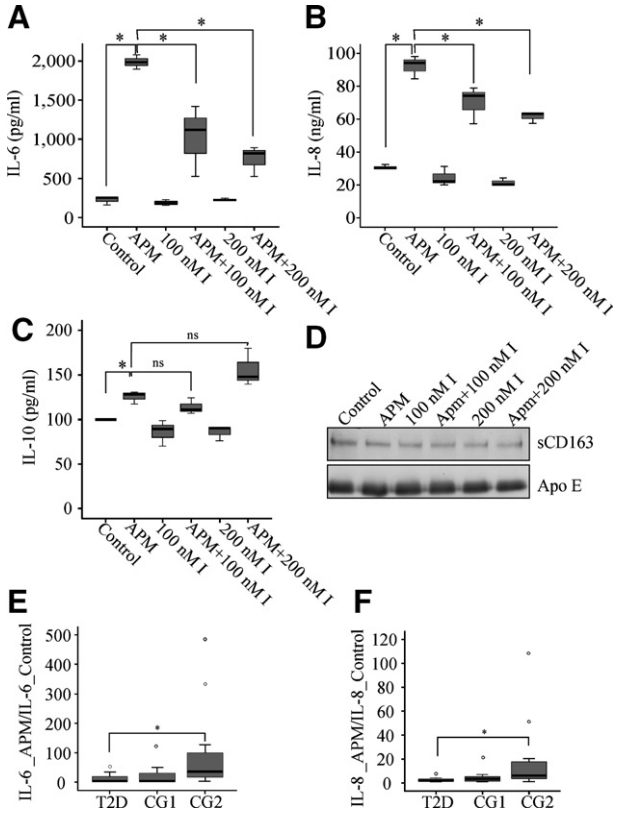

Fig. 3. Adiponectin-mediated induction of IL-6 and IL-8 depends on the p38 MAPK and is reduced in monocytes of type 2 diabetes patients. (A) Monocytes of three healthy individuals were incubated with 10 μg/ml adiponectin, with 100 nM or 200 nM p38 MAPK inhibitor, or with adiponectin in combination with 100 nM or 200 nM p38 MAPK inhibitor and IL-6 (A), IL-8 (B), IL-10 (C), soluble (s) CD163 and ApoE (D) were determined in the supernatants. (E) Monocytes of type 2 diabetes patients (T2D), overweight controls (CG1) and normal-weight controls (CG2) were incubated with medium alone, or medium and 10 μg/ml adiponectin for 24 h and adiponectin-induced IL-6 relative to control-cultivated monocytes is given. The circles indicate outliers. (F) Monocytes of type 2 diabetes patients (T2D), overweight controls (CG1) and normal-weight controls (CG2) were incubated with medium alone or medium and 10 μg/ml adiponectin for 24 h and adiponectin-induced IL-8 relative to control-cultivated monocytes is given. The circles indicate outliers. $^{*}P < 0.05$, non significant (ns).

To find out whether the inhibitory effects of the inhibitor were specific the release of IL-10, apolipoprotein E (Apo E) and soluble CD163 were determined in these supernatants. IL-10 is upregulated by adiponectin [18,19] and was also slightly induced in the monocytes studied herein. IL-10 in the supernatant of adiponectin-treated cells was 128.3% (117.0–130, $P = 0.01$ versus control) when controls were set to 100%. However, this induction was not reduced by the p38 MAPK inhibitor (Fig. 3C). Secretion of ApoE, a protein that is repressed in lipopolysaccharide stimulated macrophages [20] and soluble CD163, a macrophage specific receptor that is shedded from the cells in response to pathogen infections [21], were similar in all supernatants (Fig. 3D). In addition, the release of lactat dehydrogenase as a marker of cell death was determined and was similar in all supernatants (not shown).

3.4. Adiponectin resistance in monocytes of T2D patients

The adiponectin stimulated release of IL-6 and IL-8 was also determined in the monocytes of CG2, CG1 and T2D and the ratio of IL-6 release in adiponectin-treated cells to IL-6 secretion of cells cultivated in medium alone was calculated for monocytes of each donor separately. IL-6 induction was highest in CG2 monocytes (36.8-fold induction, range 7.7–485), tended to be lower in CG1 cells (4.1-fold, range 1.4–129.0) and was significantly lower in monocytes of T2D patients with a 4.4-fold upregulation (range 1.4–54.0, $P = 0.008$) when compared to CG2 (Fig. 3E). IL-8 upregulation was 6.8-fold (1.4–108) in CG2, 3.6-fold (1.6–21.5) in CG1 and 1.9-fold (1.5–8.0) in T2D. Whereas the difference was not significant between T2D and CG1, adiponectin-mediated increase of IL-8 was reduced in monocytes of T2D ($P = 0.004$) when compared to CG2 (Fig. 3F).

4. Discussion

Recent investigations in knock-out mice revealed that the biologic activity of adiponectin depends on the adiponectin receptors AdipoR1 and AdipoR2 [11]. Whereas several studies demonstrated that systemic adiponectin is reduced in obesity, type 2 diabetes or patients with coronary artery disease, the levels of the corresponding receptors have not been extensively studied [2,3,22]. A lower expression of AdipoR1 and AdipoR2 mRNA was described in human skeletal muscle of first degree relatives of type 2 diabetes patients [13,14], whereas a second study found a positive correlation of AdipoR1 mRNA expression in human myotubes with insulin, C-peptide, plasma triglycerides and cholesterol [23]. The expression of AdipoR2 mRNA was only associated with plasma triglyceride concentrations [23].

In the current experiments, an elevated mRNA expression of AdipoR1 and AdipoR2 was demonstrated in monocytes of overweight probands and type 2 diabetes patients. Oral medication like PPARα and PPARγ agonists, that influence adiponectin receptor 2 mRNA [12] most likely do not explain elevated expression of both receptors because, at least in the obese controls, these drugs have not been applied. The mRNA levels of AdipoR1 and AdipoR2 showed a strong positive correlation indicating a co-regulation of the receptors. AdipoR1 and AdipoR2 protein, however, were found reduced in monocytes of T2D when compared to normal-weight controls.

Therefore elevated mRNA levels seem to be associated with reduced protein abundance although this has to be verified in a larger study cohort.

In vitro, adiponectin induced AdipoR1 and AdipoR2 mRNA and protein in monocytes of normal-weight controls but not of T2D patients. Systemic adiponectin did not correlate with monocytic AdipoR2 mRNA and there was only a weak positive correlation with monocytic AdipoR1 mRNA indicating that circulating adiponectin may not represent a major regulator of monocytic adiponectin receptor mRNA levels in vivo. AdipoR1 mRNA positively correlated to the WHR, and therefore, soluble factors influenced by obesity and/or visceral fat mass, besides adiponectin, may modulate monocytic expression of AdipoR1.

Adiponectin receptor mRNA and protein have not been compared in circulating monocytes of probands with impaired and normal glucose tolerance so far. Alberti et al. determined the mRNAs of these receptors in lymphocytes of obese individuals and the abundance of AdipoR1 and AdipoR2 in lymphocytes of normal glucose tolerant donors tended to be reduced when compared to lymphocytes of impaired glucose tolerant donors or T2D patients [24]. In addition, similar to our data, a positive correlation of AdipoR1 mRNA with systemic adiponectin was identified [24]. Several studies have demonstrated that adiponectin reduces the release of proinflammatory cytokines in LPS-activated monocytes, and thus, it was initially described as an antiinflammatory molecule [7,25]. In contrast, adiponectin also induced IL-6, IL-8 and MCP-1 in non-activated monocytes, and therefore, is more properly regarded as an immuno-modulatory factor [8–10]. In the current experiments, we demonstrated that the enhanced secretion of IL-6 and IL-8 in adiponectin treated monocytes was associated with elevated IL-6 and IL-8 mRNA levels. Inhibition of the p38 MAPK abrogated adiponectin-mediated release of IL-6 and IL-8 indicating that adiponectin activates the p38 MAPK signaling pathway in monocytes. Adiponectin-mediated induction of IL-6 and IL-8 was reduced in monocytes of T2D patients and tended to be lower in monocytes of the obese donors. Therefore the influence of adiponectin is impaired in monocytes of T2D patients and this may be explained by the reduced abundance of AdipoR1 protein that was demonstrated to mediate adiponectin-stimulated activation of the p38 MAPK and subsequent IL-6 release at least in fibroblasts [16].

To summarize, the current experiments indicate that monocytes of type 2 diabetes patients have reduced AdipoR1 and AdipoR2 protein and this may explain the impaired response to adiponectin-mediated activation of the p38 MAPK signalling pathway.

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